Jpn. J. Cancer Res. 82, 835–840, July 1991

Nuclear p53 Immunoreaction Associated with Poor Prognosis of Breast Cancer

Keiichi Iwaya,[1,2] Hitoshi Tsuda,[1] Hoshio Hiraide,[2] Kuniyoshi Tamaki,[2] Syouetsu Tamakuma,[2] Takashi Fukutomi,[3] Kiyoshi Mukai[1] and Setsuo Hirohashi[1,4]

[1]Pathology Division, National Cancer Center Research Institute and [3]Department of Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 and [2]First Department of Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa-shi, Saitama 359

p53 protein has been frequently detected at high levels in the nuclei of human breast cancer cells. We analyzed immunohistochemically the association between nuclear localization of p53 protein and clinical and histological parameters of breast cancer patients. Surgically resected tissues of 73 primary breast cancers were processed by acetone fixation and paraffin embedding and examined using an anti-p53 monoclonal antibody, PAb1801. p53 immunoreactivity was detected in the nuclei of cancer cells in 17 cases (23%). The nuclear p53 immunoreaction was closely associated with overexpression of c-*erb*B-2 protein ($P<0.05$), high histologic grade ($P<0.01$), advanced clinical stage ($P<0.05$), and negative estrogen receptor status ($P<0.01$). When 31 cases which had been followed up for more than 50 months were examined, a positive nuclear p53 immunoreaction was found to be significantly associated with shorter overall survival of patients ($P<0.01$). These results suggest that immunohistochemical examination of nuclear p53 protein is clinically useful as an indicator of breast cancer aggressiveness.

Key words: Human breast cancer — Tumor suppressor gene — p53 — Immunohistochemistry — Prognostic factor

Detection of biologically aggressive breast cancer is important for prediction of relapse-free or overall survival of patients and for choosing an adjuvant systemic therapy effective for delaying the relapse of overt cancer. At present, TNM clinical stage and/or status of lymph nodal metastasis are the predominant prognostic factors. However, other parameters have also been shown to be significant prognostic indicators, e.g., hormone receptor status, histologic grade,[1-3] and DNA ploidy pattern in primary breast cancer tissue.[4,5] Extensive studies on genetic alterations in cancer cells at the DNA level and immunohistochemical investigations using specific monoclonal and polyclonal antibodies have revealed that several alterations in oncogenes and the levels of expression of their products could be potentially significant prognostic indicators. For example, amplification of the c-*erb*B-2 and c-*myc* proto-oncogenes and overexpression of their products are known to be associated with aggressive clinical behavior of breast cancer.[6-10]

Inactivation or abnormal expression of tumor suppressor genes is also suggested to play an important role in human cancer development. One of these tumor-suppressor genes, p53 gene, codes for 53-kDa nuclear protein.[11] Mutation of the p53 gene at a highly conserved sequence and alteration in the expression of p53 protein are frequently present in human malignancies.[12-14] Immunohistochemically, nuclear p53 localization has also been shown frequently in various types of human cancers.[13-16] In human breast cancer cell lines, mutation of the p53 gene and altered expression of its protein are common events, and p53 protein has been detected immunohistochemically in the nuclei of cancer cells.[14] In this study, we examined the association between nuclear p53 immunoreaction and the prognosis of patients with human breast cancer in order to clarify the clinical significance of examining p53.

MATERIALS AND METHODS

Patients We investigated a total of 73 primary breast cancers from 31 patients who had undergone radical or modified radical mastectomy at the National Defense Medical College Hospital, Tokorozawa, Saitama, between 1986 and 1987, and 42 patients who had undergone similar operations at the National Cancer Center Hospital, Tokyo, in 1990. Each tumor was divided into four parts: (1) formalin-fixed paraffin-embedded tissue for routine histopathological diagnosis, and for immunohistochemical study of c-*erb*B-2 protein expression, (2) fresh frozen tissue for examination of estrogen receptor (ER)[5] status using enzyme-linked immunosorbent assay, (3) acetone-fixed paraffin-embedded tissue processed by the AMeX (acetone, methyl benzoate, xylene) method,[17] which was thereafter stored at 4°C until immunohistochemical study of p53 protein, and (4) stock tissue frozen at −80°C until required for assay. The

[4] To whom correspondence should be addressed.

[5] Abbreviations used: ER, estrogen receptor; PBS, phosphate-buffered saline.

clinical stage of the cancer at the time of surgery was defined according to the TNM system. Histologic grading of the primary tumor was performed according to a system[18] based on a modification of the WHO classification.[19]

The 31 patients in the first series of this study ranged in age from 26 to 73 years (mean, 49.1 yr). Of these patients, 29 had undergone curative surgery, and two had had palliative surgery because of distant metastasis. Breast cancer recurred in 9 of the patients (29%) and four (13%) died due to metastases during the follow-up period. Tumor sizes on palpation (largest dimension) ranged from 1.0 to 8.0 cm. For all patients, follow-up data for periods longer than 50 months were available. In the 42 cases in the second series, the patients ranged in age from 29 to 72 years, and tumor size on palpation ranged from 1.0 to 11 cm. For all 42 cases, curative surgery was performed. Information on ER status was available in 61 cases.

Immunohistochemistry An antibody against p53 protein, PAb1801, was used for immunohistochemistry. PAb1801 (Oncogene Science, Inc., Manhasset, NY), is a murine monoclonal antibody against human p53, recognizing a denaturation-resistant epitope between amino acids 32 and 79 (Banks *et al.* 1986[20]), and has been shown to recognize both the wild-type and mutant forms of p53 protein. AMeX-processed paraffin-embedded cancer tissue was cut into 5-μm sections, deparaffinized in xylene and rehydrated in acetone. Sections were post-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, washed in water, and preincubated in 2% normal swine serum in PBS. The sections were then incubated at room temperature overnight with monoclonal antibody. PAb1801 was used at a dilution of 1:100. After being washed with PBS, they were incubated for 30 min with biotinylated horse anti-mouse immunoglobulin as a secondary antibody (Vector Laboratories Inc., Burlingame, CA) diluted 1:200. Subsequently, they were incubated for 30 min with avidin-biotinyl-peroxidase complex using a Vectastain ABC kit (Vector) diluted 1:100. The peroxidase reaction was performed using 0.02% 3,3′-diaminobenzidine tetrahydrochloride-hydrogen peroxide as a chromogen, and 0.01 *M* sodium azide was used as an endogenous peroxidase inhibitor in Tris buffer (pH 7.6) for 5–10 min. Nuclear counterstaining was performed with 1% methyl green (Chroma, Kongen, Germany). Between each step, the slides were washed three times (5 min each) in PBS.

The results of the immunoreaction were the same between frozen sections and AMeX sections in each case of the first series, indicating the applicability of AMeX sections for detection of p53 by PAb1801.

Formalin-fixed paraffin-embedded tissue was also cut into 5-μm-thick sections and subjected to immunohistochemical analysis of c-*erb*B-2 expression according to the method described previously, using a polyclonal antibody against c-*erb*B-2 protein[21] (Nichirei Inc., Higashimurayama, Tokyo). Criteria for judgment of the c-*erb*B-2 immunoreaction have been described previously.[21] We judged that c-*erb*B-2 protein was overexpressed when immunoreaction was strongly positive (++), and we judged that it was not overexpressed when immunoreaction was weakly positive (+) or negative(−).

Statistical analysis Association of p53 expression with the number of metastatic lymph nodes, tumor size on palpation, overexpression of c-*erb*B-2 protein and ER status was calculated by using the chi-squared test. Curves for overall survival were drawn according to the Kaplan-Meier method,[22] and differences between the curves were analyzed by applying the generalized Wilcoxon test.[23]

RESULTS

A distinct nuclear immunoreaction for p53 was judged as positive. Seventeen (23%) of the 73 cases showed a positive immunoreaction with PAb1801 (Fig. 1). Positive cells were distributed evenly in the cancerous tissue, and there were 3 cases of 50–60% of positivity in tumor cells, 9 of 70–80%, and 5 of more than 90% in the 17 positive cases. In the positive cells, the nuclear staining pattern was diffuse. Cytoplasmic staining was seen in a few cases, but its intensity was very weak.

The number of cases showing a nuclear p53 immunoreaction with PAb1801 was significantly larger among those with overexpression of c-*erb*B-2 protein ($P<0.05$), those of histologic grade 3 ($P<0.01$), those with negative ER status ($P<0.01$), and those at clinical stage III or IV ($P<0.05$). There was no significant association between nuclear p53 immunoreaction and tumor size on palpation, or lymph node status. However, the incidence of p53-positive cases was relatively high (40%) among those with tumors 5.0 cm or larger (Table I).

There was a strong association among overexpression of c-*erb*B-2 protein, nuclear p53 immunoreaction and histologic grade of breast cancer. Overexpression of c-*erb*B-2 protein and/or nuclear p53 immunoreaction were detected in 14 (56%) of 25 grade 3 cases, but in only 12 (28%) of 43 grade 2 cases and in none (0%) of five grade 1 cases (Table II).

In the first series, the nuclear p53 immunoreaction was positive in three (75%) of four patients who died of breast cancer, whereas only one of 26 patients died among the negative cases. There was a significant difference in the overall survival curves between the group with a positive p53 immunoreaction detected by PAb-1801 and the other group with no p53 immunoreaction

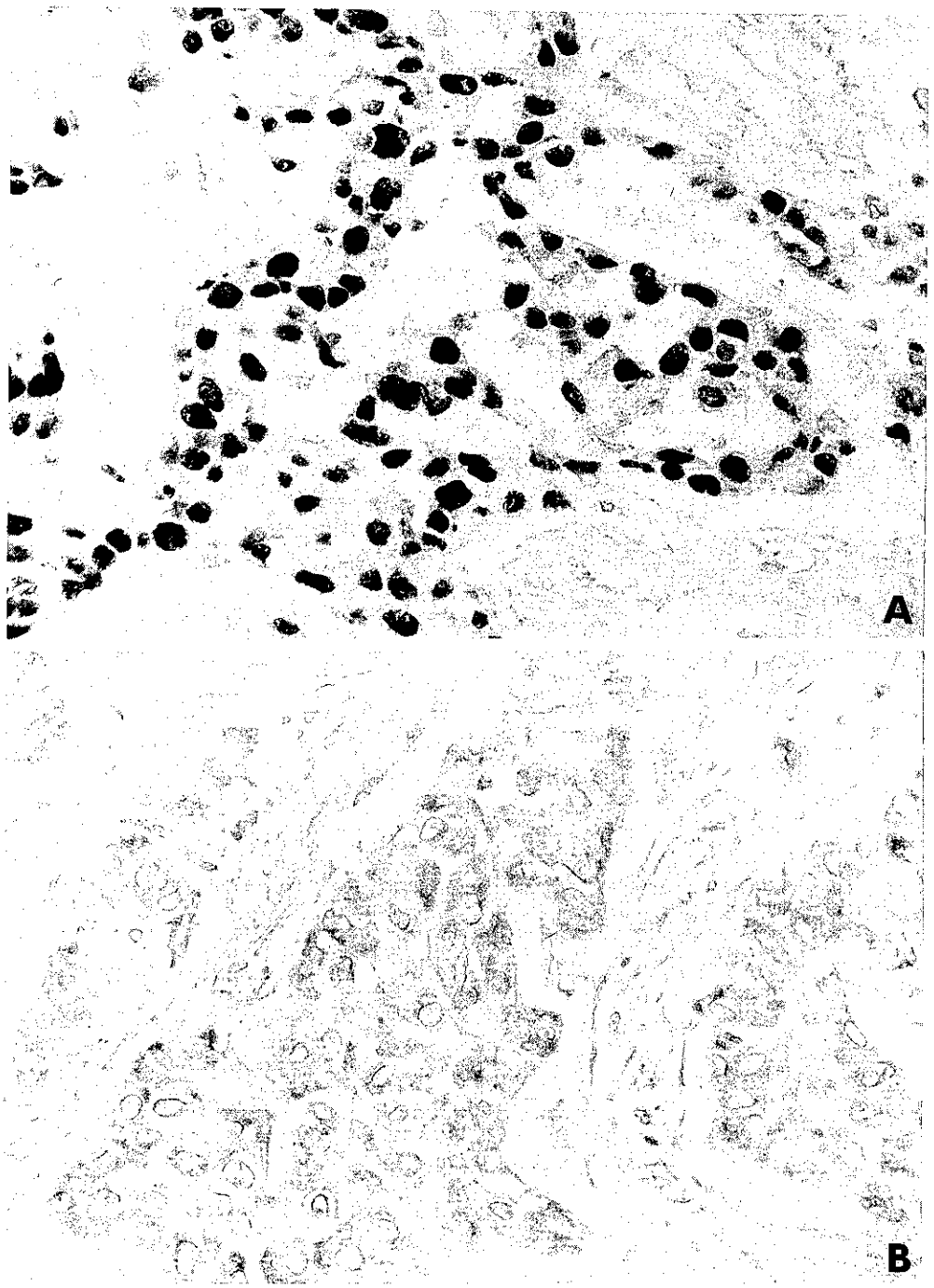

Fig. 1. Photomicrograph of primary breast carcinoma stained by p53 monoclonal antibody, PAb1801. A: Positive case. Nuclear staining is distributed diffusely and evenly. B: Negative case. No nuclear staining is detectable.

(Fig. 2, $P<0.01$). However, there was no significant difference between the disease-free survival curves when the two p53-positive patients who had undergone palliative surgery were excluded from the analysis.

DISCUSSION

The clinical significance of p53 expression has already been examined in Caucasian patients with breast cancer.

Table I. Correlation between p53 Nuclear Staining with PAb1801 and Clinical and Histological Parameters in Breast Cancer Patients

	p53 immunoreactivity, number of cases (%)			*P* value
	−	+	total	
A. Axillary lymph node status				NS
no metastasis	27 (77)	8 (23)	35	
metastasis	29 (76)	9 (24)	38	
B. Tumor size				NS
<5 cm	50 (79)	13 (21)	63	
>5 cm	6 (60)	4 (40)	10	
C. Overexpression of c-*erb*B-2 protein				$P<0.05$
positive	11 (56)	9 (44)	20	
negative	47 (90)	6 (10)	53	
D. Estrogen receptor				$P<0.01$
positive	35 (90)	4 (10)	39	
negative	11 (50)	11 (50)	22	
E. Stage				$P<0.05$
I	16 (100)	0 (0)	16	
II	28 (74)	10 (26)	38	
III	11 (65)	6 (35)	17	
IV	0 (0)	2 (100)	2	
F. Histologic grade				$P<0.01$
1	5 (100)	0 (0)	5	
2	38 (88)	5 (12)	43	
3	13 (52)	12 (48)	25	

NS, not significant.

Table II. Association among Nuclear p53 Immunoreaction, Overexpression of c-*erb*B-2 Protein and Histologic Grade of 73 Breast Carcinomas

Nuclear p53 immunoreaction and overexpression of c-*erb*B-2	Number of cases (%)		
	Histologic grade		
	1	2	3
p53 positive c-*erb*B-2 overexpressed	0 (0)	2 (5)	7 (28)
p53 positive c-*erb*B-2 not overexpressed	0 (0) 0 (0)	2 (5) 12 (28)	4 (16) 14 (56)
p53 negative c-*erb*B-2 overexpressed	0 (0)	8 (18)	3 (12)
p53 negative c-*erb*B-2 not overexpressed	5 (100)	31 (72)	11 (44)
Total	5 (100)	43 (100)	25 (100)

Crawford *et al.* first detected circulating antibody against p53 in sera of 9%[24] of cancer patients. They found subsequently that 24% of breast carcinomas show nuclear p53 protein immunohistochemically.[25] Recent immunohistochemical studies have found a correlation between p53 immunoreaction and clinical or histological parameters of breast cancer, e.g., negative ER status, positive epidermal growth factor receptor status, high

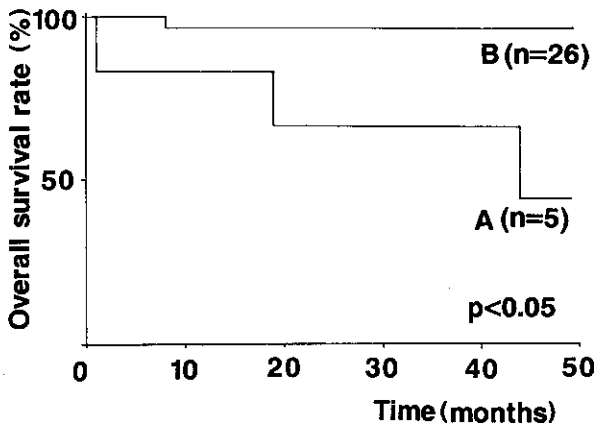

Fig. 2. Kaplan-Meier overall survival curves for patients with breast carcinoma with regard to the immunoreaction with PAb-1801. A: Positive cases (5 cases). B: Negative cases (26 cases).

histologic grade of tumor, and high Ki-67 score.[15] In Japanese patients, we were able to confirm that nuclear p53 immunoreaction was more frequent in aggressive breast cancers, e,g., those with overexpression of c-*erb*B-2 oncoprotein, histologic grade 3, advanced clinical stage, and negative ER status. Our data also suggested that immunohistochemistry for p53 protein using acetone-fixed paraffin-embedded sections would be clinically useful for predicting the prognosis of patients.

Generally, the nuclear p53 immunoreaction is considered to reflect nuclear accumulation of mutant p53 protein, which is coded by the mutated form of the p53 gene and has a prolonged half-life. The mutant p53 protein has been shown to form a complex with heat-shock protein (HSP70) in experiments using a human cancer cell line.[26, 27] Furthermore, in studies of lung, breast, and colorectal cancer cell lines and tissues, most of the cancers showing the nuclear p53 immunoreaction were also found to carry a mutation of the p53 gene.[13, 14, 16] From these findings and our present ones, it is suggested that breast cancer showing a positive nuclear p53 immunoreaction carries the mutated p53 gene, and that mutation of p53 may play an important role as a mechanism for aggressive biological behavior of human breast cancer.

Both nuclear p53 immunoreaction and overexpression of c-*erb*B-2 protein were frequently detected in histologic grade 3 breast cancers, which have a poor prognosis. A high histologic grade of breast cancer is determined by the presence of structural and nuclear atypia and an increased number of mitotic figures in cancer cells. Therefore, alterations in p53 and c-*erb*B-2 protein are suggested to be involved in loss of differentiation, formation of nuclear polymorphism and a coarse chromatin pattern, and/or rapid growth of breast cancer.

ACKNOWLEDGMENTS

The authors are grateful to Mr. S. Furuya for excellent technical assistance, to Drs. T. Hirota, M. Itabashi, H. Yamamoto, T. Nanasawa, K. Hirata, Y. Moriya, T. Naruke, and K. Hojo for providing surgical specimens, and to the staff of the photocenter for photography.

(Received March 5, 1991/Accepted May 7, 1991)

REFERENCES

1) Bloom, H. J. G. and Richardson, W. W. Histological grading and prognosis in breast cancer. *Br. J. Cancer,* **11,** 359–377 (1957).
2) Freedman, L. S., Edwards, D. N., McConnell, E. M. and Downham, D. Y. Histologic grade and other prognostic factors in relation to survival of patients with breast cancer. *Br. J. Cancer,* **40,** 44–55 (1979).
3) Fisher, E. R., Gregorio, R. M. and Fisher, B. The pathology of invasive breast cancer: a syllabus derived from findings of National Surgical Adjuvant Breast Project (protocol no. 4). *Cancer,* **36,** 1–85 (1975).
4) Clark, G. M., Dressler, L. G., Owens, M. A., Pounds, G., Oldaker, T. and McGuire, W. L. Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. *N. Engl. J. Med.,* **320,** 627–633 (1989).
5) Coulson, P. B., Thorthwaite, J. R., Woolley, T. W., Sugarbker, E. V. and Seckinger, D. Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer patient's survival. *Cancer Res.,* **44,** 4187–4196 (1984).
6) Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science,* **235,** 177–182 (1987).
7) Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, T., Tsugane, S., Yamamoto, H., Miyajima, N., Toyoshima, K., Yamamoto, T., Yokota, J., Yoshida, T., Sakamoto, H., Terada, M. and Sugimura, T. Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units; *hst*-1/*int*-2 and c-*erb*B-2/*ear*-1. *Cancer Res.,* **49,** 3104–3108 (1989).
8) Guérin, M., Barrois, M., Terrier, M-J., Spielmann, M. and Riou, G. Overexpression of either c-*myc* or c-*erb*B-2/*neu* protooncogenes in breast carcinomas: correlation with poor prognosis. *Oncogene Res.,* **3,** 21–31 (1988).
9) Barnes, D. M., Lammie, G. A., Millis, R. R., Gullick, W. L., Allen, D. S. and Altman, D. G. An immunohisto-

chemical evaluation of c-*erb*B-2 expression in human breast carcinoma. *Br. J. Cancer,* **58,** 448–452 (1988).
10) Van de Vijver, M. J., Peterse, J. L., Mooi, W. J., Wisman, P., Lomans, J., Dalesio, O. and Nusse, R. *Neu*-protein overexpression in breast cancer: association with comedo-type ductal carcinoma *in situ* and limited prognostic value in stage II breast cancer. *N. Engl. J. Med.,* **319,** 1239–1245 (1988).
11) Lane, D. P. and Crawford, L. V. T antigen is bound to a host protein in SV40-transformed cells. *Nature,* **278,** 261–263 (1979).
12) Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C. and Vogelstein, B. Mutations in the p53 gene occur in diverse human types. *Nature,* **342,** 705–708 (1989).
13) Bartek, J., Iggo, R., Gannon, J. and Lane, D. P. Genetic and immunochemical analysis of mutant p53 in breast cancer cell line. *Oncogene,* **5,** 893–899 (1990).
14) Rodrigues, N. R., Rowan, A., Smith, M. E. F., Kerr, I. B., Bodmer, W. F., Gannon, J. V. and Lane, D. P. p53 mutation in colorectal cancer. *Proc. Natl. Acad. Sci. USA,* **87,** 7555–7559 (1990).
15) Cattoretti, G., Rilke, R., Andreola, S., D'Amato, L. and Delia, D. p53 expression in breast cancer. *Int. J. Cancer,* **41,** 178–183 (1988).
16) Iggo, R., Gatter, K., Bartek, J., Lane, D. and Harris, A. L. Increased expression of mutant form of p53 oncogene in primary lung cancer. *Lancet,* **335,** 675–679 (1990).
17) Sato, Y., Mukai, K., Watanabe, S., Goto, M. and Shimosato, Y. The AMeX method; a simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. *Am. J. Pathol.,* **125,** 431–435 (1986).
18) Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, T., Tsugane, S., Watanabe, S., Terada, M. and Yamamoto, H. Correlation between histologic grade of malignancy and copy number of c-*erb*B-2 gene in breast carcinoma. *Cancer,* **65,** 1794–1800 (1990).
19) "Histological Typing of Breast Tumors," 2nd Ed. (1981). World Health Organization, Geneva.
20) Banks, L., Matlaski, G. and Crawford, L. Isolation of human-p53-specific monoclonal antibodies and their use in the study of human p53 expression. *EMBO J.,* **159,** 529–534 (1986).
21) Tsuda, H., Hirohashi, S., Shimosato, Y., Tanaka, Y., Hirota, T., Tsugane, S., Shiraishi, M., Toyoshima, K., Yamamoto, T., Terada, M. and Sugimura, T. Immunohistochemical study on overexpression of c-*erb*B-2 protein in human breast cancer: its correlation with gene amplification and long-term survival of patients. *Jpn. J. Cancer Res.,* **81,** 327–332 (1990).
22) Kaplan, E. L. and Meier, P. Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.,* **53,** 457–481 (1958).
23) Gehan, E. A generalized Wilcoxon test for comparing arbitrarily singly censored samples. *Biometrika,* **52,** 203–224 (1956).
24) Crawford, L. V., Pim, D. C. and Bulbrook, R. D. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. *Int. J. Cancer,* **30,** 403–408 (1982).
25) Crawford, L. V., Pim, D. C. and Lamb, P. The cellular protein p53 in human tumors. *Mol. Biol. Med.,* **2,** 261–272 (1984).
26) Stur, H. W. S., Chumakov, P., Welch, W. J. and Jenkins, J. R. Mutant p53 proteins binding hsp72/73 cellular heat shock-related proteins in SV40-transformed monkey cells. *Oncogene,* **1,** 201–211 (1987).
27) Finlay, C. A., Hinds, P. W., Tan, T-H., Eliyahu, D., Oren, M. and Levin, A. J. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.,* **7,** 531–539 (1988).